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## THE USE OF PLASMA METABOLITES TO PREDICT WEEKLY BODY-MASS CHANGE IN RED KNOTS

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**Abstract.** The Red Knot (*Calidris canutus*) is a long-distance migrant breeding on tundra in the high Arctic and wintering along temperate and tropical coasts. Preflight fueling rate is a major determinant of successful migration, yet individual fueling rates are impossible to determine because Red Knots cannot be recaptured easily. These problems can be overcome by estimating changes in body mass from plasma metabolites. Plasma metabolites are, however, sensitive to stress and time since last meal, limiting studies to situations where birds can be bled almost immediately after capture. Such sampling is almost impossible in the field, where Red Knots are often captured with mist nets in darkness. This study on captive Red Knots investigates whether plasma metabolites obtained from blood samples taken up to 3 hr after capture can be used to predict individual long-term (weekly) body-mass changes during the natural spring preflight fueling period. Triglyceride decreased and  $\beta$ -hydroxybutyrate increased with time since capture, and these changes varied with time since start of the spring fueling period.  $\beta$ -Hydroxybutyrate and uric acid were correlated with weekly body-mass change, but triglyceride was not. Triglyceride was correlated with overall body mass. Weekly body-mass change was best predicted with a model including all metabolites and body mass. Time of blood sampling (immediately or 3 hr after capture) did not affect the accuracy of the predictions. The predictions were not accurate enough to allow comparisons of individuals; they should be used only to compare groups.

**Key words:** body-mass change, *Calidris canutus*, fueling rate, plasma metabolite, Red Knot, shorebird, triglyceride, uric acid,  $\beta$ -hydroxybutyrate

### Uso de Metabolitos Plasmáticos para Predecir Cambios Semanales del Peso Corporal en *Calidris canutus*

**Resumen.** Los individuos de la especie *Calidris canutus* migran largas distancias, se reproducen en la tundra a altas latitudes en el Ártico e invernán a lo largo de las costas templadas y tropicales. La tasa de abastecimiento antes del vuelo es la principal determinante del éxito de migración. Sin embargo, estas tasas de abastecimiento son imposibles de determinar debido a que los individuos de *C. canutus* no son recapturados con facilidad. Estos problemas pueden ser minimizados con la estimación de los cambios en el peso corporal a partir de metabolitos plasmáticos. Estos metabolitos plasmáticos, sin embargo, son sensibles al estrés y al tiempo desde la última comida, lo que limita los estudios a aquellas situaciones en las que las muestras de sangre pueden ser tomadas inmediatamente después de la captura. Este tipo de muestreos en el campo son casi imposibles, debido a que los individuos de *C. canutus* son capturados generalmente con redes de neblina durante la noche. Este estudio con individuos en cautiverio de *C. canutus* investigó si los metabolitos plasmáticos obtenidos de muestras de sangre tomadas hasta 3 horas después de la captura pueden ser usados para predecir los cambios en el largo plazo (semanales) del peso corporal de los individuos durante el periodo natural de abastecimiento de primavera, que ocurre antes del vuelo. Los triglicéridos disminuyeron y el  $\beta$ -hidroxibutirato aumentó con el tiempo desde la captura, y estos cambios variaron con el inicio del periodo de engorde de primavera. El  $\beta$ -hidroxibutirato y el ácido úrico se correlacionaron con los cambios semanales en el peso corporal, pero los triglicéridos no. Los triglicéridos se correlacionaron con el peso corporal general. La mejor forma de predecir el cambio semanal en el peso corporal fue con un modelo que incluyó todos los metabolitos y el peso corporal. El tiempo de colecta de la muestra de sangre (inmediatamente o 3 horas después de la captura) no afectó la precisión de las predicciones. Las predicciones no fueron lo suficientemente precisas para permitir la comparación entre individuos; estas deberán usarse sólo para comparar grupos de individuos.

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## INTRODUCTION

The Red Knot (*Calidris canutus*), a shorebird, is a long-distance migrant that breeds on tundra in the high Arctic and winters along temperate and tropical coasts. Its migration routes range in length from 5000 to 15 000 km and are covered in two or more long nonstop flights, depending on subspecies (Piersma and Davidson 1992, Piersma et al. 2005, Piersma 2007). Successful migration depends to a large extent on pre-flight fueling rate, which affects the duration of preflight preparations (e.g., stopover duration), fuel load at departure, time required for migration, body condition at arrival, and indirectly reproduction and survival (Alerstam and Hedenström 1998, Alerstam et al. 2003). Yet, determining individual fueling rates in free-living Red Knots is difficult because recapturing individuals within the same season is almost impossible (Piersma et al. 2005) and investigating whether fueling rates relate to direct and indirect components of fitness such as subsequent survival or reproductive success remains problematic (but see Morrison et al. 2007).

Plasma metabolites may provide a means of overcoming these problems, because they can be used to estimate individual rates of body-mass change in free-living birds from a single blood sample (Jenni-Eiermann and Jenni 1994, Jenni and Schilch 2001, Schaub and Jenni 2001, Guglielmo et al. 2002, Seaman et al. 2005, Cerasale and Guglielmo 2006a, Smith et al. 2007). In these studies, body-mass change was best estimated from levels of triglyceride (indicator of fat deposition),  $\beta$ -hydroxybutyrate (indicator of fat catabolism), and/or uric acid (indicator of protein catabolism). In the Red Knot these metabolites also reflect seasonal changes in metabolic processes, body mass, and rate of body-mass change, although triglyceride levels (uncorrected for glycerol) are not correlated with rate of body-mass change (Jenni-Eiermann et al. 2002).

So far, plasma-metabolite concentrations have been used to predict body-mass changes over intervals of hours or one to two days in small passerines and shorebirds (Jenni-Eiermann and Jenni 1994, Williams et al. 1999, Cerasale and Guglielmo 2006a). Only Williams et al. (1999), in the Western Sandpiper (*Calidris mauri*), used longer intervals (7 days) and showed that at this scale relationships were less consistent (glycerol) and even nonexistent (triglyceride and  $\beta$ -hydroxybutyrate) than at the shorter intervals (1–2 days). Because short-term fasting affects metabolite levels, research has also been limited to situations where blood samples can be taken within a few minutes after the bird's capture (Jenni-Eiermann and Jenni 1994, Williams et al. 1999, Cerasale and Guglielmo 2006a).

In contrast to that of passerines, the fueling period of shorebirds before migration often takes several weeks (Zwarts et al. 1990). Therefore, in shorebirds estimates of individual body-mass and fueling trajectories might better be examined over longer intervals, such as a week. Because capturing shorebirds with mist nets at night makes it almost impossible to bleed birds within minutes of their being captured, it would

be helpful if metabolites in blood taken several hours after capture still yield robust predictions of body-mass change. Capitalizing on the preflight fueling period for northward migration (i.e., spring preflight fueling), which occurs naturally and is maintained for several years by captive Red Knots even when day length is held constant (Piersma et al. 1996, Jenni-Eiermann et al. 2002, Piersma 2002, Piersma et al. 2008), we investigated if plasma metabolites from blood taken some hours after capture can be used to estimate body-mass change over the previous week in captivity. After peak mass is reached, captive Red Knots lose their surplus body stores via a gradual body-mass decrease. We took blood samples during this whole spring fueling cycle of body-mass increase and decrease.

The study focuses on the following questions: (1) Do metabolite levels vary with time since capture? (2) Are metabolite levels correlated with long-term (weekly) body-mass change? (3) Can long-term body-mass change be predicted from metabolite levels of blood samples obtained 3 hr after capture, and is this prediction comparable with the prediction from metabolites of blood samples obtained immediately after capture?

## METHODS

### ANIMALS

Red Knots of subspecies *islandica*, breeding in North America and wintering in Europe, caught in the Dutch Wadden Sea between 1994 and 2000, were kept in groups in outdoor aviaries under natural light and temperature conditions at the Royal Netherlands Institute for Sea Research (NIOZ, Texel, The Netherlands). In each aviary (3 m long  $\times$  2 m wide  $\times$  2 m high), knots had access to a small barren artificial mudflat in which they could probe. The floor was continuously flushed with fresh sea water to prevent foot diseases. Food (trout pellets, Trouvit Classic 2P, Skretting, Hendrix SpA, Italy; composition: crude protein 45%, carbohydrate 21%, crude fat 16%, crude ash 9%, lysine 3%, indigestible fibers 2%, phosphorus 1%) and fresh water were available *ad libitum*. This high-fat, high-protein diet was used in a previous study (Jenni-Eiermann et al. 2002) and resembles the standard diet used in comparable studies of captive Western Sandpipers (e.g., Williams et al. 1999, Seaman et al. 2005). As part of the birds' routine care, their body mass ( $\pm$  1 g) was determined weekly. One day after the day of caretaking, blood samples were taken and body mass was determined. From these data, weekly change in body mass was calculated as (body mass at first blood sample – body mass on day of caretaking the week before) per (number of days between measurements) ( $\text{g day}^{-1}$ ), enabling the calculation of weekly body-mass change for the first sampling day of the experiment. There were 8 days between body-mass measurements [with three exceptions: there were once 6 days (2001), 7 days, and 9 days (2003) between measurements].

The variation in metabolite levels was followed during two separate episodes of preflight fueling in spring. In 2001, blood samples were taken weekly of one group of 11 Red Knots (8 males, 1 female, 2 of unknown sex; on average 3.6 years in captivity; 8 birds were on average 3.4 years old, 3 were adults at capture) from 18 May to 27 June, the latter half of the spring fueling cycle. In 2003, 14 Red Knots (8 males and 6 females; on average 4.9 years in captivity; 12 birds were on average 5.2 years old, 2 were adults at capture), divided into two groups, were sampled weekly from 8 April to 22 July, covering the whole spring fueling cycle. One bird (K388) was used in both years. The experiment complied with the Dutch Law on Experimental Animals and was approved by the Experimental Animals Ethics Committee (approval number DEC-2000.04, both years).

#### BLOOD SAMPLING

We took blood samples (ca. 180  $\mu$ l) every 7 days [with three exceptions: there were once 5 days (2001), 6 days, and 8 days (2003) between measurements] by puncturing the wing vein and drawing the blood into heparinized capillaries. The capillaries were kept on ice until centrifuged (12 min at 6900 g). Plasma samples were stored in a freezer ( $-20^{\circ}\text{C}$ ) until transport to the Swiss Ornithological Institute in Switzerland for analysis.

In 2001, Red Knots were sampled four times on each sampling day. For the first sample, the knots were captured between 10:30 and 10:55 (sample  $T_0$ ). After sampling, the knots were kept for 1 hr in a transport box to mimic capture stress in the field. Then the second blood sample was taken (sample  $T_1$ ) and the birds were released in the aviary, where water was present but food was not, to mimic the short-term fast under field-capture conditions. The third sample ( $T_3$ ) was taken 3 hr after sample  $T_0$ , after which the birds were returned to the aviary. This sampling time is similar to general practice in our field situations. The final sample ( $T_6$ ) was taken 6 hr after sample  $T_0$ , after which the birds were returned to the aviary and food was provided. Samples  $T_0$ ,  $T_3$ , and  $T_6$  were taken on average 4.5 min after we entered the aviary ( $n = 204$ ; range 0.5–17 min), and sample  $T_1$  was taken on average 4.3 min after we opened the transport box ( $n = 69$ ; range 0.8–12 min).

In 2003, sampling was limited to twice per sampling day, at  $T_0$  and  $T_3$ .  $T_1$  was excluded because it is uncommon that in the field Red Knots are sampled around 60 min after capture.  $T_6$  was excluded because preliminary analysis of the 2001 data indicated that levels of metabolites at  $T_6$  deviated considerably from those at  $T_0$  and were little correlated with body-mass change. Further catching, keeping, and sampling procedures were similar to those in 2001. For the  $T_0$  samples, the Red Knots were captured between 10:37 and 11:10. Three hours thereafter, sample  $T_3$  was taken. Samples were taken on average 2.9 min after we entered the aviary ( $n = 416$ ; range 0.8–8.7 min).

#### METABOLITE ANALYSES

The concentrations of the metabolites were determined by means of standard diagnostic kits.  $\beta$ -Hydroxybutyrate was analyzed by an enzymatic test (2001: Sigma Diagnostics procedure no. 310-UV; 2003: Wako Diagnostics, cyclic enzymatic method), triglycerides by a colorimetric test (2001: Sigma Diagnostics GPO-Trinder, no. 337; 2003: Wako Diagnostics GPO-DAOS method), modified for small amounts of plasma. We measured triglycerides by analyzing first the concentration of free glycerol and then, in a second step, the concentration of glycerol bound with free fatty acids by hydrolysis with lipoprotein lipase. Additionally, in 2003 uric acid was analyzed (Wako, enzymatic FDAOS method). All analyses were done in duplicate. The levels of triglycerides we present are corrected for glycerol, i.e., with glycerol subtracted from the overall triglyceride levels.

#### STATISTICAL ANALYSES

The metabolite data were normally distributed after transformation by a natural logarithm (Kolmogorov–Smirnov test per sampling time, SPSS 14.0). Further references to metabolite concentrations refer to the transformed data. The data have a nested structure with observations (four or two per day, once per week) nested within individuals, and individuals nested within years. Therefore we used generalized linear mixed models (GLMMs; Snijders and Bosker 1999) to determine if the data varied with time since capture (i.e., from sample  $T_0$  to sample  $T_6$ ), within the experimental period, or between years.

The data of 2001, with four samples per day, were used to determine if metabolites varied with time since capture and if this variation was constant over the spring fueling cycle. A similar analysis was run on body mass. For each individual and day, linear regressions were fitted through the data of body mass,  $\beta$ -hydroxybutyrate, or triglyceride versus time (SPSS 14.0). Regressions were not fitted when all four sampling points were not available. This situation occurred especially in week 1, when body mass data for  $T_0$  and  $T_1$  were missing. Such cases have been assigned as missing. Next, to partition the variation in the slopes of the regressions among and within individuals, we ran a GLMM with individual and observation (nested within individual) fitted as random effects and the constant as the only fixed effect (null model). From this null model we derived the average slope  $\pm$  SE for the average individual under average circumstances. To test if the effect of time since capture varied over the experimental period, the null model was expanded to a full model by including week (continuous variable; fixed effect) and its square (to model nonlinear relationships). Backward elimination of nonsignificant terms ( $P > 0.05$ ) was used as a criterion for model selection (Crawley 1993). Analyses of GLMMs were performed with MLwiN 2.02 (Rabash et al. 2004).



Keeping Red Knots under a natural cycle of light and dark synchronizes their spring preflight fattening periods by year (see Piersma et al. 2008), so day after 1 April (1 April = April day 0) was used as unit of time. To determine if years differed, we used data from approximately the same period: in 2001 April days 48–88 (all seven days of measurement) and in 2003 April days 43–93 (8 of the 16 days of measurement). From the bird used in both years (K388) the data of 2001 were excluded to avoid pseudoreplication. With only two years available, the between-year variance could not be estimated accurately, and year was entered as a categorical fixed effect in the GLMM analysis (Rabash et al. 2004). For each of the dependent variables (body mass, weekly body-mass change,  $\beta$ -hydroxybutyrate, and triglyceride), we fitted first a full model with individual identity and observation nested within individuals as random effects and April day (linear and quadratic component) and year as fixed effects. The final model was obtained via a stepwise backward analysis, excluding at each step the nonsignificant fixed effect with the highest  $P$ -value ( $P > 0.05$ ). The GLMM was limited to  $T_0$  and  $T_3$  (separately analyzed) because these points of sampling were used in both 2001 and 2003. Note that data on weekly body-mass change were available for  $T_0$  only.

To determine if metabolite levels were correlated with long-term (weekly) body-mass change, linear regressions were calculated for each metabolite and sampling time, with weekly body-mass change as independent variable. The relationship between metabolites and body mass was also analyzed. We used the 2003 data, the most complete dataset, for this analysis. Next, we calculated predictive relationships for  $T_0$  and  $T_3$  separately, using a backward multiple-regression analysis with weekly body-mass change as dependent and metabolites and body mass as independent variables (SPSS 14.0, note the change in dependent variable). Since uric acid was not measured in 2001, we also calculated partial prediction models without uric acid. To determine how well weekly body-mass change was estimated, and if this differed by sampling time, we subtracted estimated weekly body-mass change from measured weekly body-mass change for both years and plotted this difference versus measured weekly body-mass change. Linear regressions were fitted through the data and sampling times were compared with univariate analysis of variance (SPSS 14.0). Means are presented  $\pm$  SE. For all analyses the significance level  $\alpha$  was set at 0.05.

## RESULTS

### DO METABOLITES VARY WITH TIME SINCE CAPTURE AND TIME SINCE START OF THE SPRING PREFLIGHT FATTENING PERIOD?

Body mass decreased with time since first sample ( $T_0$ ), i.e., time since capture (data of 2001; constant estimate of the null model, i.e., the mean slope of the average individual under average circumstances, was  $-0.472 \pm 0.035$ ,  $P < 0.001$ ). This decrease

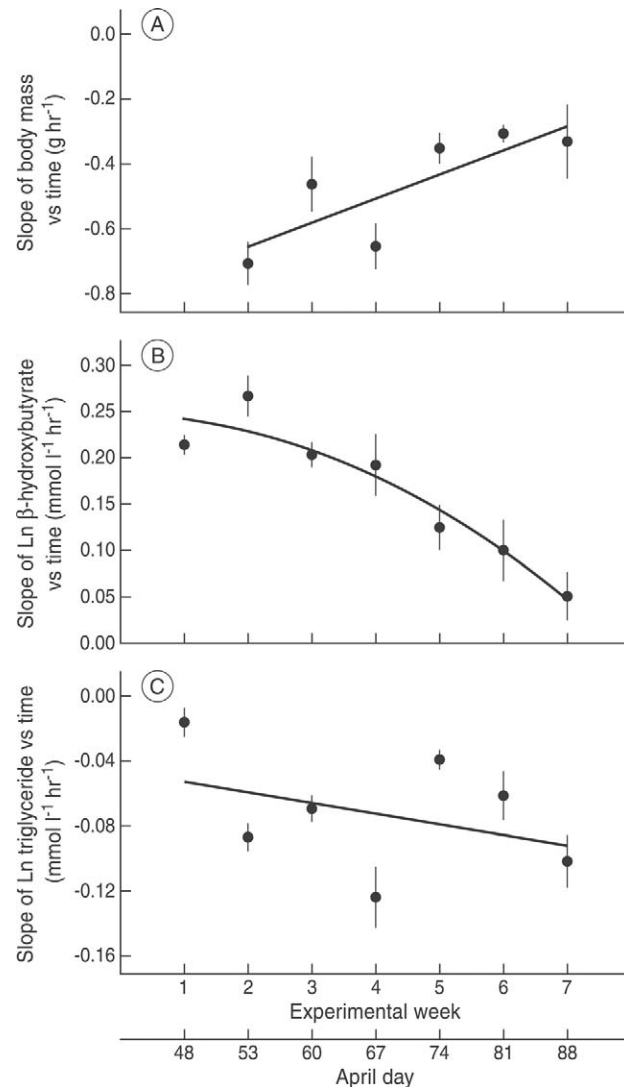


FIGURE 1. The mean slope of the individual linear regressions of (a) body mass, (b)  $\beta$ -hydroxybutyrate, and (c) triglyceride versus time since first sample, versus time. The lines represent the results of the final models of the GLMM analyses: (a) body mass =  $-0.806 (\pm 0.087) + 0.075 (\pm 0.018)$  week,  $\chi^2 = 17.0$ ,  $df = 1$ ,  $P < 0.001$ ; (b)  $\beta$ -hydroxybutyrate =  $0.246 (\pm 0.016) - 0.004 (\pm 0.001)$  week<sup>2</sup>,  $\chi^2 = 59.1$ ,  $df = 1$ ,  $P < 0.001$ ; and (c) triglyceride =  $-0.046 (\pm 0.013) - 0.007 (\pm 0.003)$  week,  $\chi^2 = 5.0$ ,  $df = 1$ ,  $P < 0.05$ . The second x axis gives the appropriate April days for the experimental weeks (note that April day 0 is 1 April). This axis is not linear, as there were only 5 days between week 1 and 2. Parameters for the GLMMs are given in Table 1.

varied linearly with time of year, becoming smaller at the end of the experiment when body mass approached normal summer levels (Fig. 1a and 2a). The short-term fast also had an effect on the metabolites.  $\beta$ -Hydroxybutyrate concentrations increased with time since capture (mean slope  $0.165 \pm 0.012$ ,  $P < 0.001$ ), and, just like the change in body mass, this change decreased with time, though in a quadratic, not linear, way (Fig. 1b). Triglyceride concentrations decreased with time since capture

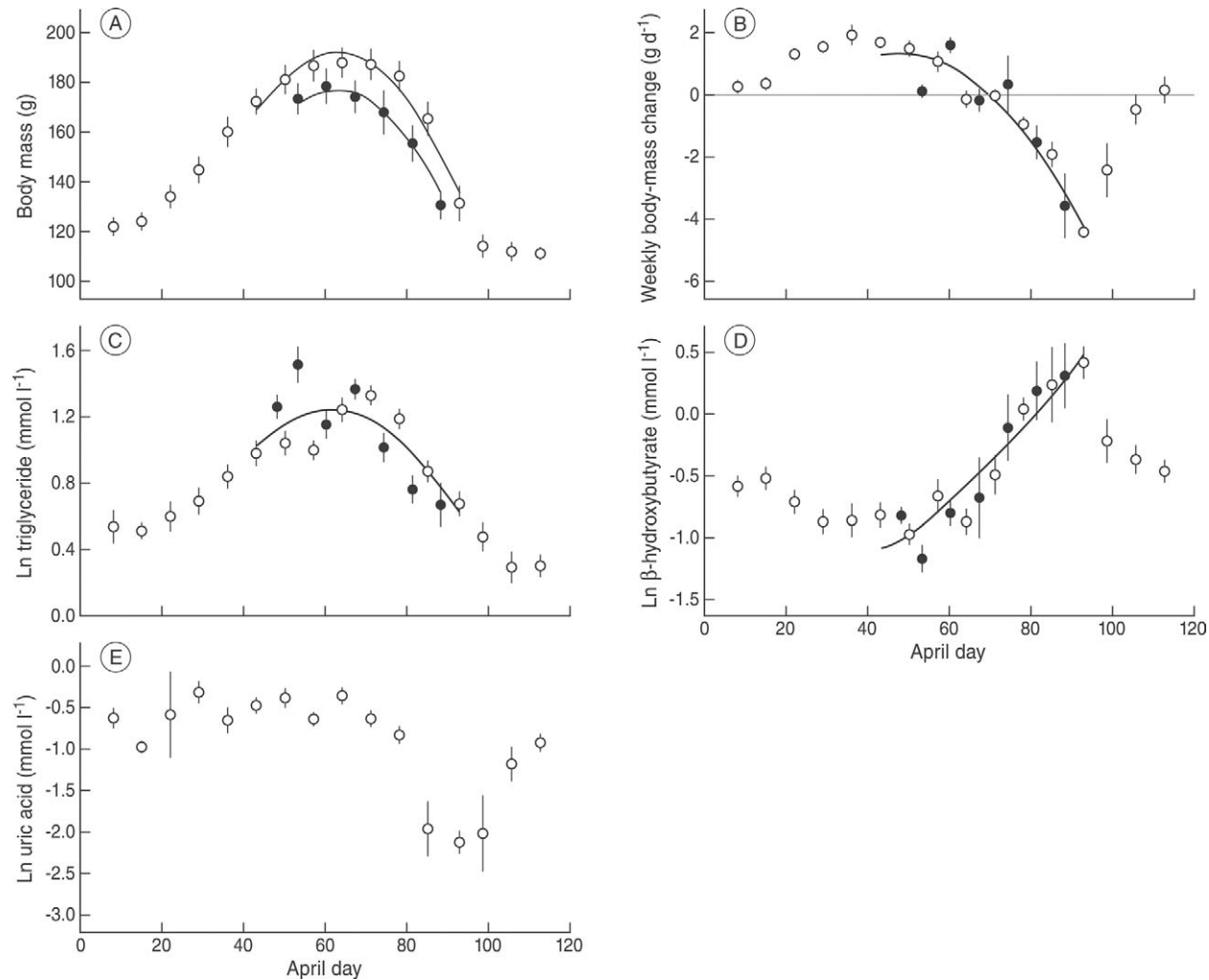


FIGURE 2. Mean (a) body mass, (b) weekly body-mass change, (c) triglyceride, (d)  $\beta$ -hydroxybutyrate, and (e) uric acid determined at  $T_0$  in 2001 (black circles) and 2003 (white circles) versus time. The solid lines represent the results of the final models of the GLMM analyses of the comparison of the two years: (a) body mass =  $-58.89 (\pm 25.57) + 15.41 (\pm 7.49) \text{ year} + 7.58 (\pm 0.74) \text{ April day} - 0.06 (\pm 0.01) \text{ April day}^2$ ,  $\chi^2 = 233.8$ ,  $df = 3$ ,  $P < 0.001$ ; (b) weekly body-mass change =  $-4.42 (\pm 2.46) + 0.25 (\pm 0.07) \text{ April day} - 0.003 (\pm 0.001) \text{ April day}^2$ ,  $\chi^2 = 224.5$ ,  $df = 2$ ,  $P < 0.001$ ; (c) triglyceride =  $-1.15 (\pm 0.45) + 0.08 (\pm 0.01) \text{ April day} - 0.0006 (\pm 0.0001) \text{ April day}^2$ ,  $\chi^2 = 74.0$ ,  $df = 2$ ,  $P < 0.001$ ; (d)  $\beta$ -hydroxybutyrate =  $-1.52 (\pm 0.12) + 0.00023 (\pm 0.00002) \text{ April day}^2$ ,  $\chi^2 = 130.6$ ,  $df = 1$ ,  $P < 0.001$ . When year was significant, separate lines for each year are given. The dotted line in (b) indicates no change in body mass. Please note that uric acid was not determined in 2001. April day 0 is 1 April.

(mean slope  $-0.072 \pm 0.006$ ,  $P < 0.001$ ). In contrast to those of body mass and  $\beta$ -hydroxybutyrate, this change increased linearly with time of year (Fig. 1c; the slope becomes more negative with time). In all cases, the between-individual effect in the null model was very small and not significant ( $\chi^2$  test,  $df = 1$ , all  $P > 0.05$ ), implying that almost all of the variance was due to within-individual effects. Because all parameters varied significantly with time since capture, all further analyses were done for the sampling times separately, thereby limiting the analyses to the sampling times available for both years,  $T_0$  and  $T_3$ .

Body mass increased with time until peak masses were reached, whereupon it decreased again toward normal summer

levels (Fig. 2a and 3a). At  $T_0$ , body mass was slightly higher in 2003 than in 2001, but note that this difference, despite considerable statistical power, was only weakly significant ( $\chi^2 = 3.9$ ,  $df = 1$ ,  $P = 0.05$ ). At  $T_3$ , the year effect disappeared ( $\chi^2 = 3.5$ ,  $df = 1$ ,  $P = 0.06$ ).

Weekly body-mass change was initially positive and increased slowly (Fig. 2b). When body mass approached its peak, weekly body-mass change decreased and became increasingly negative. Just before normal summer body masses were reached, the negative weekly body-mass change abruptly decreased toward zero. Weekly body-mass change in the two years did not differ ( $\chi^2 = 0.2$ ,  $df = 1$ ,  $P = 0.69$ ).

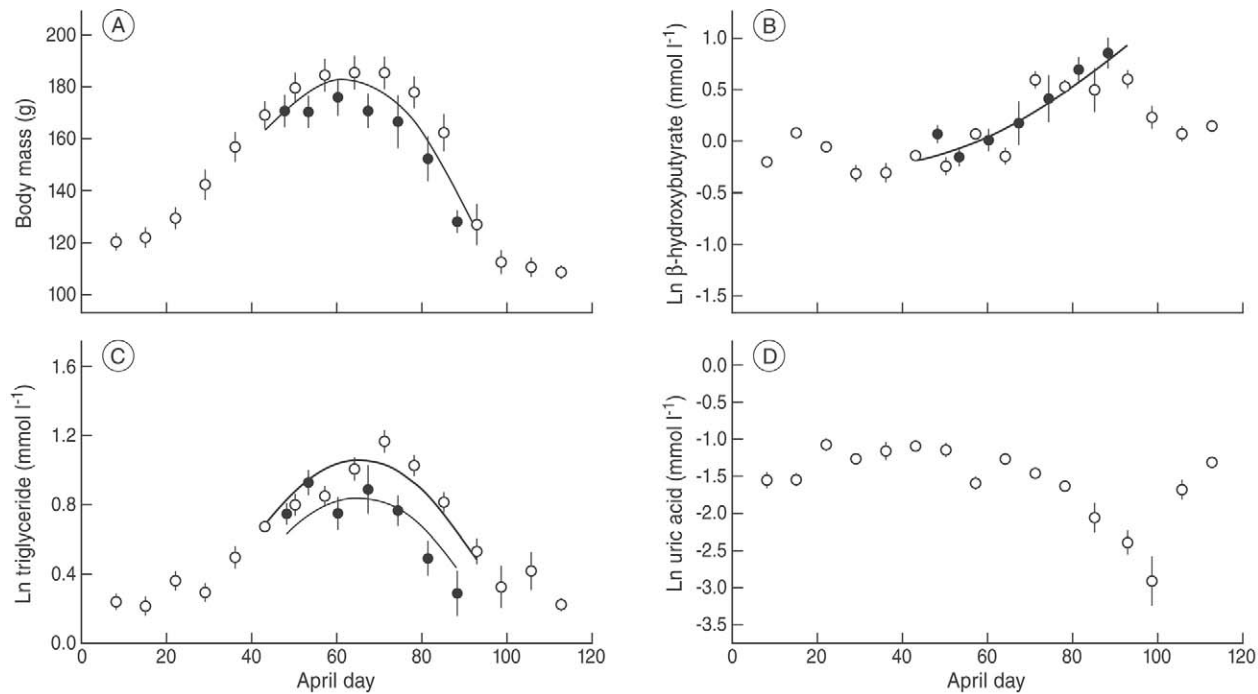


FIGURE 3. Mean (a) body mass, (b)  $\beta$ -hydroxybutyrate, (c) triglyceride, and (d) uric acid determined at  $T_3$  in 2001 (black circles) and 2003 (white circles) versus time. The solid lines represent the results of the final models of the GLMM analyses of the year comparison: (a) body mass =  $-39.81 (\pm 23.62) + 7.27 (\pm 0.71)$  April day  $- 0.06 (\pm 0.01)$  April day<sup>2</sup>,  $\chi^2 = 251.0$ ,  $df = 2$ ,  $P < 0.001$ ; (b)  $\beta$ -hydroxybutyrate =  $-0.46 (\pm 0.08) - 0.00015 (\pm 0.00001)$  April day<sup>2</sup>,  $\chi^2 = 107.6$ ,  $df = 1$ ,  $P < 0.001$ ; (c) triglyceride =  $-2.38 (\pm 0.41) + 0.22 (\pm 0.07)$  year +  $0.10 (\pm 0.01)$  April day  $- 0.0008 (\pm 0.0001)$  April day<sup>2</sup>,  $\chi^2 = 87.3$ ,  $df = 3$ ,  $P < 0.001$ . When year was significant, separate lines for each year are given. Please note that uric acid was not determined in 2001 and that weekly body-mass change was determined only for  $T_0$ . April day 0 is 1 April.

The pattern of  $\beta$ -hydroxybutyrate concentrations with time was negatively associated with the pattern of weekly body-mass change (Fig. 2b, d, and 3b). At  $T_3$ , the resemblance to the pattern of weekly body-mass change was less pronounced than at  $T_0$ .  $\beta$ -Hydroxybutyrate did not differ by year ( $\chi^2 = 0.01$ ,  $df = 1$ ,  $P = 0.94$ ; and  $\chi^2 = 0.7$ ,  $df = 1$ ,  $P = 0.40$ , for  $T_0$  and  $T_3$ , respectively).

The patterns of triglyceride concentration with time were remarkably similar to that of body mass and showed no resemblance to the pattern of weekly body-mass change (Fig. 2b, c and 3c). Triglyceride concentrations did not differ by year at  $T_0$  ( $\chi^2 = 0.1$ ,  $df = 1$ ,  $P = 0.72$ ), but at  $T_3$ , concentrations were higher in 2003 ( $\chi^2 = 9.8$ ,  $df = 1$ ,  $P < 0.001$ ).

The patterns of uric acid concentration with time also resembled the pattern of weekly body-mass change (Fig. 2b, e, and 3d). In contrast to that of  $\beta$ -hydroxybutyrate, this resemblance became more obvious at  $T_3$ .

#### DO METABOLITES RELATE TO BODY MASS AND WEEKLY BODY-MASS CHANGE?

We explored the relationships between the metabolites and body mass or weekly body-mass change by using the data of 2003, the year in which the whole spring preflight fattening period was sampled. Triglyceride increased with body mass at  $T_0$  and  $T_3$  (Fig. 4a and b); body mass explained more than half

of the variance in triglyceride. Triglyceride concentrations were not correlated with weekly body-mass change at  $T_0$  (Fig. 5a), but at  $T_3$  a significant decrease was found (Fig. 5b). Note, however, that weekly body-mass change explained only 1.6% of the variance in triglyceride concentration at  $T_3$ .

$\beta$ -Hydroxybutyrate decreased linearly with weekly body-mass change, though this decrease was not significant at  $T_3$  ( $P = 0.07$ ; Fig. 5c and d). At  $T_0$ ,  $\beta$ -hydroxybutyrate also decreased with body mass (Fig. 4c), but only 2.6% of the variance was explained by body mass while 44.4% was explained by weekly body-mass change. At  $T_3$ ,  $\beta$ -hydroxybutyrate was not correlated with body mass (Fig. 4d).

Uric acid increased with weekly body-mass change and body mass at both  $T_0$  and  $T_3$ , but weekly body-mass change explained much more of the variance (52.6 and 58.2%, versus 8.1 and 9.0%, respectively; Fig. 4e, 4f, 5e, and 5f).

#### HOW WELL DO METABOLITES PREDICT WEEKLY BODY-MASS CHANGE?

Triglyceride concentrations hardly correlated with weekly mass change (Fig. 5a, b) but rather with body mass (Fig. 2 and 3). Previous studies, however, have shown that after control for body mass, triglyceride is often positively correlated with body-mass change (Williams et al. 1999, Cerasale and Guglielmo 2006a). Therefore, we calculated a series of full prediction

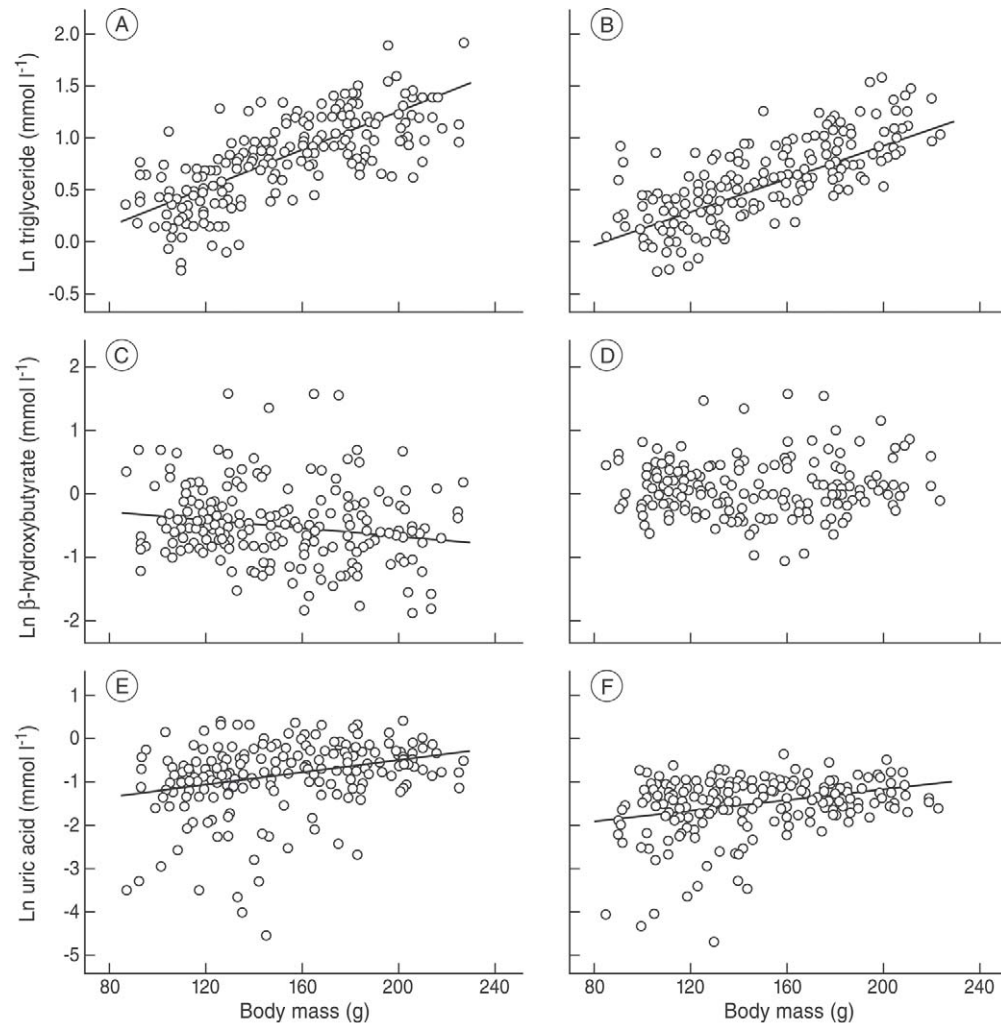


FIGURE 4. Relationships between the metabolites and body mass determined at  $T_0$  (left panels) or at  $T_3$  (right panels). Only data of 2003 are presented. Solid lines indicate the following significant linear regressions for  $T_0$ : (a)  $\ln \text{triglyceride} = -0.52 (\pm 0.09) + 0.009 (\pm 0.001) \text{ body mass}$ ,  $n = 206$ ,  $R^2 = 0.54$ ,  $P < 0.001$ ; (c)  $\ln \beta\text{-hydroxybutyrate} = -0.06 (\pm 0.19) - 0.003 (\pm 0.001) \text{ body mass}$ ,  $n = 205$ ,  $R^2 = 0.03$ ,  $P = 0.02$ ; (e)  $\ln \text{uric acid} = -1.92 (\pm 0.25) + 0.007 (\pm 0.002) \text{ body mass}$ ,  $n = 204$ ,  $R^2 = 0.08$ ,  $P < 0.001$ ; and for  $T_3$ : (b)  $\ln \text{triglyceride} = -0.67 (\pm 0.08) + 0.008 (\pm 0.001) \text{ body mass}$ ,  $n = 205$ ,  $R^2 = 0.56$ ,  $P < 0.001$ ; (f)  $\ln \text{uric acid} = -2.42 (\pm 0.20) + 0.006 (\pm 0.001) \text{ body mass}$ ,  $n = 205$ ,  $R^2 = 0.09$ ,  $P < 0.001$ .

models for  $T_0$  and  $T_3$  separately, starting with a model that included only  $\beta$ -hydroxybutyrate and uric acid, after which it was extended with triglyceride, then with body mass also. We calculated a similar series of partial prediction models excluding uric acid.

$\beta$ -Hydroxybutyrate and uric acid were good predictors of weekly body-mass change; together they explained 58% of the variation in body-mass change (Table 1). Together with body mass, they were always included in the final full prediction models. Triglyceride was also significant, except at  $T_3$  when body mass was excluded from the model. Weekly body-mass change was correlated negatively with  $\beta$ -hydroxybutyrate and triglyceride, positively with uric acid and body mass. The full prediction model explained about 58–66% of the variation. The partial models yielded similar results, except that

triglyceride was not significant without body mass in the model. The partial prediction models explained 44–48% of the variation at  $T_0$  and 34–49% of that at  $T_3$ .

Using full and partial prediction models, thereby excluding models that included initially triglyceride but not body mass, we estimated weekly body-mass change for  $T_0$  and  $T_3$  in 2003. The difference between measured and estimated weekly body-mass change was plotted against measured weekly body-mass change (Fig. 6 presents examples of these relationships). This was also done for the 2001 data, with partial models only. In all cases, the difference increased linearly with weekly body-mass change. All models underestimated large negative and positive weekly body-mass changes, over- or underestimated small body-mass changes, and heavily over- or underestimated very small weekly body-mass changes (between ca.  $-0.5$  and



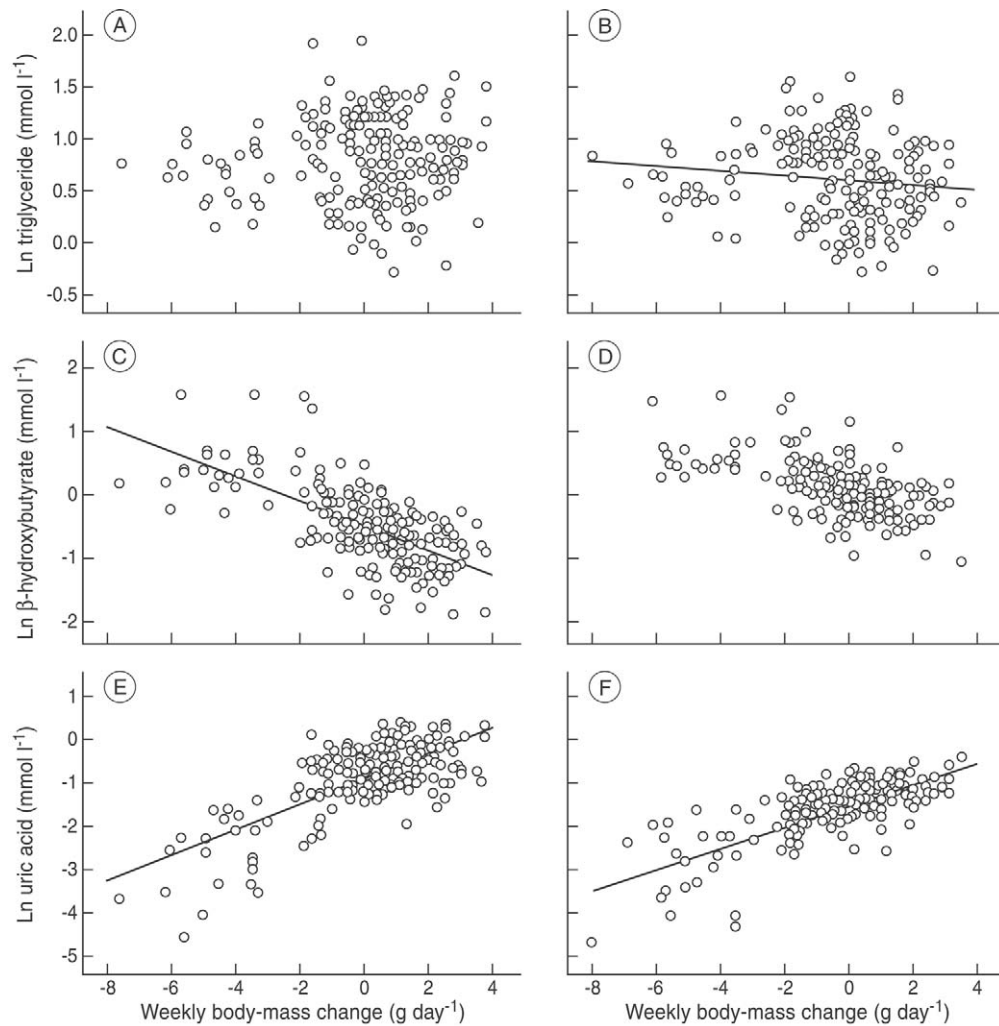


FIGURE 5. Relationships between the metabolites determined at  $T_0$  (left panels) or at  $T_3$  (right panels) and weekly body-mass change determined at  $T_0$ . Only data of 2003 are presented. Solid lines indicate the following significant linear regressions for  $T_0$ : (c)  $\ln \beta$ -hydroxybutyrate =  $-0.49 (\pm 0.03) - 0.19 (\pm 0.02)$  weekly body-mass change,  $n = 205$ ,  $R^2 = 0.44$ ,  $P < 0.001$ ; (e)  $\ln$  uric acid =  $-0.90 (\pm 0.04) + 0.30 (\pm 0.02)$  weekly body-mass change,  $n = 205$ ,  $R^2 = 0.53$ ,  $P < 0.001$ ; and for  $T_3$ : (b)  $\ln$  triglyceride =  $0.59 (\pm 0.03) - 0.02 (\pm 0.01)$  weekly body-mass change,  $n = 205$ ,  $R^2 = 0.02$ ,  $P < 0.001$ ; (f)  $\ln$  uric acid =  $-1.56 (\pm 0.03) + 0.24 (\pm 0.01)$  weekly body-mass change,  $n = 205$ ,  $R^2 = 0.58$ ,  $P < 0.001$ .

$0.5 \text{ g day}^{-1}$ ). Slopes did not differ by sampling time, except for the partial model including only  $\beta$ -hydroxybutyrate ( $P = 0.03$  and  $P = 0.04$ , for 2001 and 2003, respectively). Slopes tended to be steeper for the partial models (between 0.39 and 0.60 in 2001 and between 0.51 and 0.66 in 2003) than for the full models (0.35 and 0.41, with and without triglyceride and body mass, respectively), indicating that larger weekly body-mass changes were underestimated more by the partial models. The shallowest slopes, and thus the best predictions, were found for estimates from the full model including all metabolites and body mass. This model's estimates of weekly body-mass change were on average 45% off (45% at  $T_0$  and 46% at  $T_3$ ); very small weekly body-mass changes excluded, estimates were on average 34% off (34% at  $T_0$  and 35% at  $T_3$ ).

## DISCUSSION

Metabolite levels are sensitive to stress and time since last meal (Jenni-Eiermann and Jenni 1996, 1997, Jenni and Schilch 2001), and thus metabolite studies so far have been limited to situations where birds could be bled almost immediately after capture. Here we show for the first time that body-mass changes of Red Knots can be predicted just as well from metabolites obtained from blood samples taken 3 hr after capture as from blood samples taken immediately after capture. This is also the first evidence that in some bird species metabolite levels can predict long-term (weekly) body-mass changes. This discovery opens a new range of possible use of metabolites to estimate body-mass change in free-living wild

TABLE 1. Final prediction models of the series full and partial prediction models used to estimate weekly body-mass change. The series of full models started with  $\beta$ -hydroxybutyrate and uric acid as independent variables, adding triglyceride and body mass, respectively, in the next models. In the partial models uric acid was excluded, because it was not determined in 2001. Data of 2003 were used to fit full and partial models. Only significant parameter estimates ( $B$ ) are presented, except for the constant. The partial model with  $\beta$ -hydroxybutyrate and triglyceride as independent variables is not presented because triglyceride was not included in the final model.

Prediction model	$T_0$				$T_3$			
	$B$	$P$	Final $R^2$	$n$	$B$	$P$	Final $R^2$	$n$
Constant	$0.600 \pm 0.236$	0.01	0.58	203	$2.653 \pm 0.264$	<0.001	0.60	185
ln $\beta$ -hydroxybutyrate	$-1.089 \pm 0.208$	<0.001			$-1.557 \pm 0.241$	<0.001		
ln uric acid	$1.241 \pm 0.152$	<0.001			$1.790 \pm 0.165$	<0.001		
Constant	$1.383 \pm 0.344$	<0.001	0.60	202	$2.725 \pm 0.265$	<0.001	0.61	184
ln $\beta$ -hydroxybutyrate	$-0.990 \pm 0.208$	<0.001			$-1.472 \pm 0.244$	<0.001		
ln uric acid	$1.411 \pm 0.160$	<0.001			$1.849 \pm 0.167$	<0.001		
ln triglyceride	$-0.747 \pm 0.237$	<0.001			0.512			
Constant	$-0.344 \pm 0.498$	0.490	0.64	202	$-0.174 \pm 0.648$	0.788	0.66	184
$\beta$ -hydroxybutyrate	$-0.889 \pm 0.200$	<0.001			$-1.300 \pm 0.242$	<0.001		
ln uric acid	$1.426 \pm 0.152$	<0.001			$1.563 \pm 0.170$	<0.001		
ln triglyceride	$-1.844 \pm 0.328$	<0.001			$-1.761 \pm 0.381$	<0.001		
Body mass	$0.018 \pm 0.004$	<0.001			$0.023 \pm 0.004$	<0.001		
Constant	$-1.121 \pm 0.139$	<0.001	0.44	205	$-0.033 \pm 0.121$	0.790	0.34	186
ln $\beta$ -hydroxybutyrate	$-2.285 \pm 0.179$	<0.001			$-2.725 \pm 0.278$	<0.001		
Constant	$-3.011 \pm 0.509$	<0.001	0.49	204	$-4.109 \pm 0.580$	<0.001	0.49	185
$\beta$ -hydroxybutyrate	$-2.197 \pm 0.177$	<0.01			$-2.303 \pm 0.269$	<0.001		
ln triglyceride	$-1.223 \pm 0.379$	<0.001			$-2.253 \pm 0.460$	<0.001		
Body mass	$0.019 \pm 0.005$	<0.001			$0.036 \pm 0.005$	<0.001		

birds, which is especially important for research on migrants, provided that time between capture and blood sampling is recorded, standardized, and controlled for statistically.

#### TRIGLYCERIDES

In contrast to the findings of most studies (Jenni-Eiermann and Jenni 1994, Jenni and Schilch 2001, Cerasale and Guglielmo 2006b), the Red Knot's triglyceride concentrations at  $T_0$  were not correlated with body-mass change. Our results correspond with the findings in a previous long-term study on the Red Knot that found no correlation between triglyceride concentrations (samples taken immediately after capture) and body-mass change (Jenni-Eiermann et al. 2002). In the Western Sandpiper, residuals of triglyceride (from the relationship with body mass) are also not correlated with body-mass change (calculated over a 2-day period; Seaman et al. 2005). In an earlier study, however, residual triglyceride concentrations did correlate with short-term body-mass change but not with weekly body-mass change (Williams et al. 1999). Seaman et al. (2005) suggested that this difference might be due to the methods used to induce body-mass change: they used food restriction, whereas Williams et al. (1999) induced short-term body-mass changes via food removal and weekly body-mass change via natural variation in body mass. Thus, only a lack of food, which induced high body-mass loss and

short-term starvation, yielded a relationship between triglyceride and body-mass change, while no relationship was found when food was available. During this and the previous study on Red Knots (Jenni-Eiermann et al. 2002) food was available, which appears to confirm the suggestion that presence or absence of food during (induced) body-mass changes may have a strong effect on the relationship between triglyceride and body-mass change in shorebirds. However, this cannot explain the differences between the studies of shorebirds and those of passerines, because the latter also used food restriction rather than food removal to induce body-mass loss (Jenni-Eiermann and Jenni 1994, Jenni and Schilch 2001, Cerasale and Guglielmo 2006a, b).

Although not presented here, further analysis revealed that the correlations between metabolites and short-term body-mass change (>1 day) were comparable with those for weekly body-mass change but that short-term body-mass change explained less of the variation in metabolites (range 4–16%). Triglyceride was not correlated with short-term body-mass change at  $T_0$  and decreased with it at  $T_3$ . Hence, the lack of correlation was not due to the relatively long period over which weekly body-mass change was calculated. At  $T_0$ , final full prediction models for short-term body-mass change (starting with all metabolites and body mass) included only uric acid and body mass. At  $T_3$ , the final model included all metabolites but not body mass. The models explained much

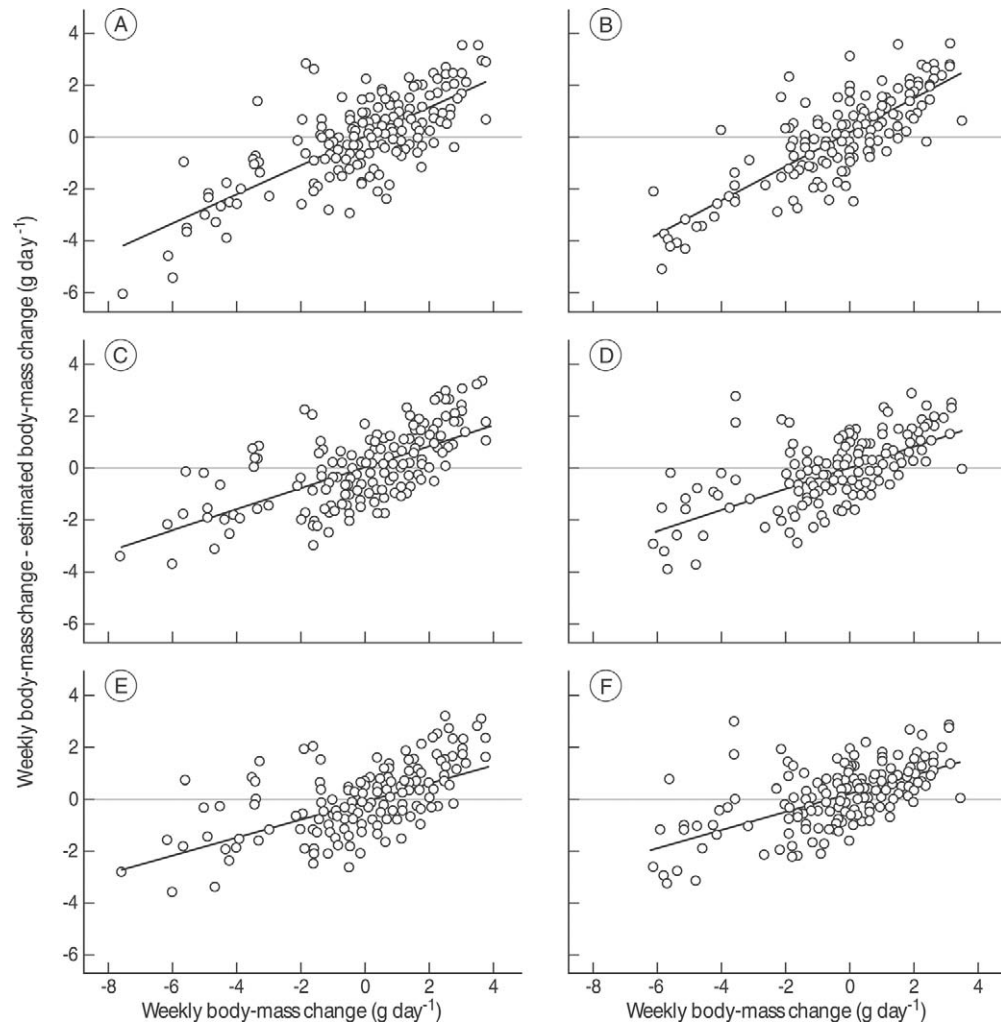


FIGURE 6. The difference between measured and estimated week body-mass change estimated via prediction models plotted against measured weekly body-mass change for  $T_0$  (left panels) and  $T_3$  (right panels) in 2003. The prediction models used included the following parameters: (a, b)  $\beta$ -hydroxybutyrate; (c, d)  $\beta$ -hydroxybutyrate and uric acid; (e, f)  $\beta$ -hydroxybutyrate, uric acid, triglyceride, and body mass. The dotted lines indicate no difference ( $Y = 0$ ). The solid lines present the linear regressions through the data: (a) difference =  $0.01 (\pm 0.07) + 0.56 (\pm 0.04)$  weekly body-mass change,  $n = 205$ ,  $R^2 = 0.56$ ,  $P < 0.001$ ; (b) difference =  $0.17 (\pm 0.07) + 0.66 (\pm 0.04)$  weekly body-mass change,  $n = 186$ ,  $R^2 = 0.66$ ,  $P < 0.001$ ; (c) and (d) difference =  $0.04 (\pm 0.05) + 0.41 (\pm 0.03)$  weekly body-mass change,  $n = 388$ ,  $R^2 = 0.41$ ,  $P < 0.001$ ; (e) and (f) difference =  $0.15 (\pm 0.07) [-0.23 (\pm 0.10) \text{ for } T_0] + 0.35 (\pm 0.02)$  weekly body-mass change,  $n = 388$ ,  $R^2 = 0.35$ ,  $P < 0.001$ .

less of the variation in body-mass change (both 18%) than the full prediction models for weekly body-mass change (60%). In Western Sandpipers, Williams et al. (1999) found that the relationships between metabolites (glycerol) and weekly body-mass change were less consistent than for short-term body-mass change. They suggested that one can find relationships between metabolites and long-term body-mass change when body-mass change is linear. The relationships then reflect a relationship with short-term body-mass change, and thus current physiological state, explaining the lower consistency. This assumption seems not to apply to the Red Knot, in which short-term body-mass change explained much less of the variation in metabolites than weekly body-mass change.

Please note, however, that in field studies on Western Sandpipers, triglyceride (corrected for body mass) was found to vary considerably with the seasons and at various sites in interesting ways, whereas  $\beta$ -hydroxybutyrate was not so informative (Guglielmo et al. 2002, Acevedo-Seaman et al 2006). Hence, one should not assume that in shorebirds in general measuring triglyceride is never worthwhile.

#### VALIDITY OF THE PREDICTIVE EQUATIONS IN THE FIELD

$\beta$ -Hydroxybutyrate and uric acid levels together explained a large part of the variation in weekly body-mass change in Red Knots. Weekly body-mass changes were best predicted by

the full predictive model including all three metabolites and body mass. Accuracy of the estimates did not differ by sampling time: estimates from metabolites from blood samples taken 3 hr after capture were as good as those from metabolites from blood samples taken immediately after capture. The estimates were not very accurate, however, especially when weekly body-mass change was very small. Therefore, predictions do not allow comparisons at the level of the individual; they should be used only to compare groups.

Despite this, the results are promising for use in the field, where often groups are compared. But there are some considerations to be made before the predictive curves are applied to field data. First, because metabolites are related to fat and protein metabolism, diet composition may affect metabolite concentrations (Seaman et al. 2005, Cerasale and Guglielmo 2006a, Smith et al. 2007). We fed the Red Knots a diet of commercial Trouvit trout pellets containing 16% fat and 45% protein. These proportions roughly resemble the fat and protein content of the tellinid bivalve *Macoma balthica*, a favorite prey of Red Knots (Zwarts and Blomert 1992, Van Gils et al. 2005), in the Dutch Wadden Sea from July to September (~10% fat and ~55% protein; Beukema and De Bruin 1977, 1979). July–September is the focal period of our field research because then *C. c. canutus* is in the Dutch Wadden Sea to fuel up for further migration toward the wintering grounds in west Africa while *C. c. islandica* arrives to molt and overwinter (Nebel et al. 2000). The resemblance between the fat and protein contents of the diets indicates that the predictive value of metabolites should be maintained in the field. Other factors that may affect metabolite levels are molt and feeding rates. In passerines, molt does not seem to affect metabolite levels (Jenni-Eiermann and Jenni 1996, 1997; but see Jenni and Jenni-Eiermann 1996). Short-term variations in feeding rate affect metabolite levels in Wilson's Warbler (*Wilsonia pusilla*). Triglyceride and  $\beta$ -hydroxybutyrate concentrations are higher and lower, respectively, at high feeding rates (mimicking a high-quality habitat) than at low feeding rates (mimicking a low-quality habitat; Zajac et al. 2006).

Preliminary analyses of metabolite samples of wild adult Red Knots (collected in the Dutch Wadden Sea in July and August 2002 and 2003) yielded an estimated mean weekly body-mass change of  $3.2 \pm 0.2$  g day<sup>-1</sup> in *C. c. canutus* ( $n = 23$ ; refueling for the second leg of its southward migration) and  $2.8 \pm 0.1$  g day<sup>-1</sup> in *C. c. islandica* ( $n = 82$ ; regaining body mass). These values are in range with estimates of refueling rate for both northward and southward migration (Nebel et al. 2000, Piersma et al. 2005).

In conclusion, metabolite levels in Red Knots are robust, enabling the use of blood samples taken up to 3 hr after capture to estimate long-term body-mass change. With the derived predictive curves, weekly body-mass changes in free-living wild Red Knots can be estimated. Given the accuracy

of the predictions, however, comparisons should be limited to the level of a group.

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